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5. INTRODUCTION:

5.1. Background and Nature of the Problem: Despite the improvement in early detection and treatment of breast cancer, the mortality rate of women with breast cancer remained high. One major obstacle for curing breast cancer is the development of **multidrug resistance** in breast cancer cells which reduces the effectiveness of chemotherapy. Breast cancer is often intrinsically drug resistant, and sometimes acquires resistance following chemotherapy. Although there are conflicting reports, **P-glycoprotein** (Pgp)-mediated multidrug resistance seems to be the most frequent mechanism of drug resistance in breast cancer (Sugawara *et al.*, 1988; Schneider *et al.*, 1989; Ro *et al.*, 1990; Goldstein *et al.*, 1989; Keith *et al.*, 1989; Salmon *et al.*, 1989; Merkel *et al.*, 1989; Sanfilippo *et al.*, 1991; Verrelle *et al.*, 1991; Wishart and Kaye, 1991; Charpin *et al.*, 1994; Bates *et al.*, 1995).

Pgp is a plasma membrane protein which functions as an efflux pump for anticancer drugs (Gottesman and Pastan, 1993). Overexpression of Pgp reduces the accumulation of cytotoxic drugs in breast cancer cells which, therefore, will survive the chemotherapeutic treatment. Pgp has been shown to interact directly with various anticancer drugs, for example, adriamycin (Bushe *et al.*, 1989), vinblastine (Cornwell *et al.*, 1986; Safa *et al.*, 1986), and colchicine (Safa *et al.*, 1989). Pgp expressed in the plasma membrane has been covalently labeled by photosensitive drug analogues (Safa *et al.*, 1987). Greenberger *et al.* (1991) and Bruggemann *et al.* (1989, 1992) suggested that the [³H]azidopine and [³H]vinblastine cross-linked to fragments including TM6 and TM12 of mouse and human Pgp, respectively. Despite the success of these drug-labelling studies, it is still not known if these domains directly bind drugs or the azido groups of these drugs are simply in a close proximity to the labelled sites. The detailed interaction between Pgp and drugs is unknown.

Using a cell-free expression system, I have previously shown that Pgp has at least two distinct topological structures in microsomal membranes (Zhang and Ling, 1991; Zhang *et al.*, 1993). Pgp molecules have also been suggested to function as chloride channels as well as drug transporters and these two functions are separable (Valverde *et al.*, 1992; Gill *et al.*, 1992; Higgins, 1992; Alternberg *et al.*, 1994). Indeed, drug transport requires ATP hydrolysis whereas the channel function can be supported by non-hydrolyzable ATP analogues (Gill *et al.*, 1992). I have previously proposed that these two functions may be carried out by the two different topological structures of the molecule; while molecules with one structure may function as a chloride channel, molecules with the other structure may function as a drug-efflux pump. However, one question remains to be answered is whether Pgp in multidrug resistant cancer cells have two alternate topologies.

5.2. Purpose of the Present Work: The current project is designed to (1) determine the topological structure of Pgp in multidrug-resistant mammalian cells and (2) map the drug-binding site in Pgp.

5.3. Methods of Approaches: To determine the topological structure of Pgp in multidrug resistant cancer cells, site-specific antibodies will be produced against relevant domains of Pgp. We will use immunocytochemistry and proteolysis/membrane protection assay to map the membrane orientation of the relevant domains of Pgp. To map the drug-binding domain,

different domains of Pgp will be expressed in bacteria in large quantity and used to study its drug binding property.

6. BODY:

6.1. Methods Used:

6.1.1. Generation and characterization of site specific antibodies:

(a), production of fusion proteins: cDNA fragments encoding relevant Pgp domains were generated using PCR technology and cloned into a bacterial expression vector (pGEX series, Pharmacia LKB Biotechnology). The final DNA products were sequenced to ensure the correct reading frame and to eliminate any possible mutations introduced by Taq DNA polymerase. The pGEX vectors provide all three possible reading frames in the multiple cloning site for generating fusion proteins with glutathione S-transferase at the N-terminus and inserted foreign protein at the C-terminus. All vectors also supply stop codons in all three reading frames for translation termination. Fusion protein was prepared from transformed bacteria JM109 and purified by separation through an affinity column of glutathione-conjugated Sepharose-4B (Smith and Johnson, 1988). The glutathione S-transferase can be cleaved off from the fusion protein using factor Xa or thrombin and removed by separation on a second glutathione-conjugated Sepharose-4B column. The purified Pgp peptides were used to raise antibodies.

(b), production of polyclonal antibodies: Rabbits were immunized subcutaneously with fusion proteins in complete Freund's adjuvant, followed by periodic boosting with peptide in incomplete Freund's adjuvant, and then bled for serum. The polyclonal antibodies were characterized against their fusion peptide antigens using ELISA or Western blot analysis (Harlow and Lane, 1988). The specificity of these antibodies for Pgp was assessed against plasma membrane fractions containing Pgp isolated from MDR cells (e.g. CH'B30 which has 10% Pgp in its total plasma membrane proteins) using Western blot and immunofluorescence. Polyclonal antibodies were affinity-purified on a MAC-25 cartridge using purified fusion peptide as previously described (Zhang and Nicholson, 1994).

6.1.2. Proteolysis/membrane protection assay of isolated membrane vesicles: Membrane vesicles were prepared from MDR cells (e.g. CH'B30) as previously described (Lever, 1977) and subjected to complete protease digestion using trypsin or proteinase K. The digested fragments were then separated on SDS-PAGE and detected by using site-specific polyclonal antibodies on Western blot. Size of the fragments resistant to protease digestion and detected by each specific antibody were analyzed according to the two alternative topologies (Zhang *et al.*, 1993).

6.1.3. Immunocytochemistry staining: The polyclonal antibodies generated in 6.1.1. were used to label fixed or fixed and permeabilized cells. The cells were fixed with paraformaldehyde (fixed, but not permeabilized) or fixed and permeabilized with acetone/methanol as previously described (Kartner *et al.*, 1985). This method of fixation and permeabilization does not affect the antigenicity of mAb C219 epitope. The labelling was detected using secondary antibody conjugated with peroxidase and the staining was viewed under a microscope.

6.1.4. Expression of a domain including TM11-TM12 of human MDR1 Pgp and drug labelling: A cDNA fragment encoding the TM11-TM12 of human MDR1 Pgp was cloned into a bacterial expression vector pET-16B and was introduced into bacteria BL21/DE3. Generation of the recombinant protein was induced by IPTG (Isopropyl β -D-Thiogalactopyranoside). Bacteria was then lysed by sonication and membrane fractions were isolated by centrifugation. The expression of the recombinant protein was determined by Western blot using monoclonal antibody C494.

6.2. Results Obtained:

6.2.1. The site-specific polyclonal antibodies generated against fusion proteins detect specifically P-glycoprotein from multidrug-resistant CHO cells We have generated site-specific polyclonal antibodies against fusion proteins containing the loop linking TM4 and TM5 (loop 4) and the loop linking TM8 and TM9 (loop 8). These antibodies are designated α Pgp-L4 and α Pgp-L8, respectively. Fig. 1 shows a Western blot of membranes isolated from sensitive and drug-resistant CHO cells detected by monoclonal antibody (mAb) C219 as well as polyclonal antibody (pAb) α Pgp-L4 and α Pgp-L8. The mAb C219 (lane 2), pAb α Pgp-L4 (lane 5) and pAb α Pgp-L8 (lane 8) specifically detected the 180-kDa Pgp. This protein was not detected in the sensitive Aux B1 cells (lanes 1, 4 and 7). It has been shown previously that hamster Pgp can be cleaved into two halves by mild protease digestion and both halves have a mAb C219 epitope (Georges *et al.*, 1991). As shown in lane 3, two half molecules of Pgp were produced by trypsin digestion and both react with the mAb C219. However, as expected, the pAb α Pgp-L4 reacted only with the N-terminal half (lane 6), whereas the pAb α Pgp-L8 reacted only with C-terminal half (lane 9) of Pgp.

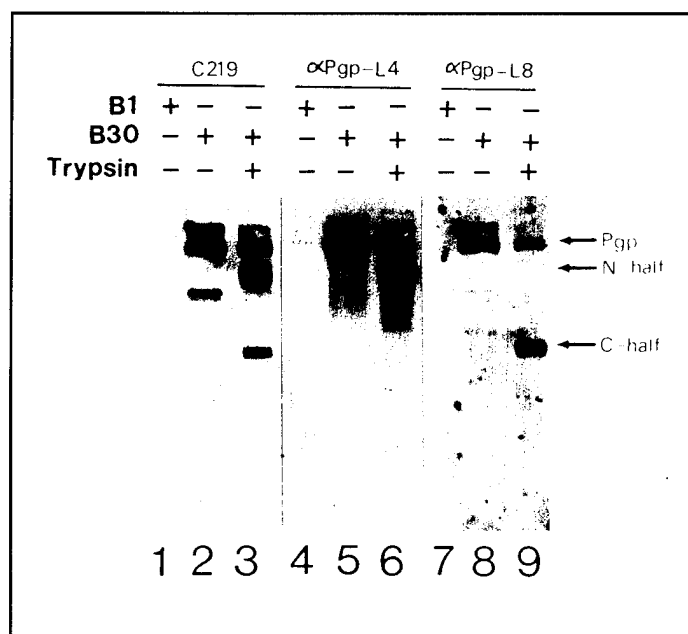


Figure 1. Characterization of the specificity of α Pgp-L4 and α Pgp-L8 antibodies to P-glycoprotein of CH^RB30 cells. Crude membranes were isolated from parental Aux B1 (lanes 1, 4 and 7) and multidrug-resistant CH^RB30 cells (lane 2, 5 and 8). Monoclonal antibody C219 as well as the polyclonal antibodies α Pgp-L4 and α Pgp-L8 specifically detected the 180-kDa Pgp and its aggregated and degraded products (lanes 2, 5 and 8). No protein from Aux B1 cells was detected by any of these antibodies. C219 detects both N- and C-terminal half fragments (lane 3). As expected, only the N-terminal half fragment was recognized by α Pgp-L4 (lane 6) whereas the C-terminal half was recognized by α Pgp-L8 (lane 9).

whereas the C-terminal half was recognized by α Pgp-L8 (lane 9).

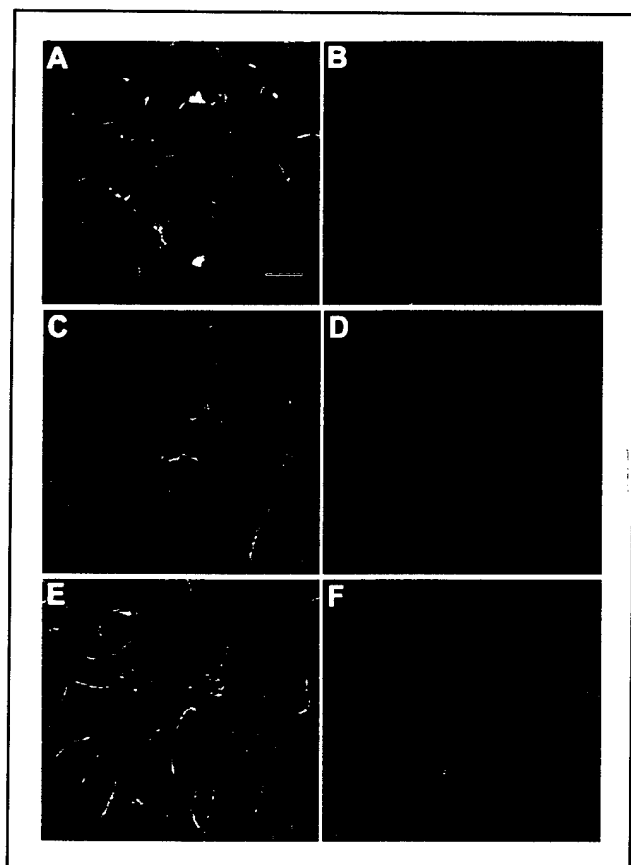


Figure 2. Immunofluorescence labelling of CH^RB30 cells using αPgp-L4 and αPgp-L8. Multidrug-resistant CH^RB30 cells were fixed and permeabilized with acetone/methanol and labelled with mAb C219 (panel A), pAb αPgp-L4 (panel C), pAb αPgp-L8 (panel E). The labeling was detected using FITC-conjugated secondary antibodies and by confocal fluorescence microscopy. In the controls, cells were labelled using normal mouse IgG (panel B) and preimmune sera of αPgp-L4 (panel D) and αPgp-L8 (panel F). All three antibodies labelled predominantly on the plasma membranes. The bar in panel A denotes 10 μm. All photographs were taken with the same magnification.

To confirm whether pAb αPgp-L4 and αPgp-L8 specifically detect Pgp on plasma membranes, we labelled fixed and permeabilized multidrug-resistant CH^RB30 cells with mAb C219, pAb αPgp-L4 and αPgp-L8, and detected the binding with

FITC-conjugated secondary antibodies and viewed on a confocal-fluorescence microscope. As shown in Fig. 2, mAb C219 (panel A), as well as pAb αPgp-L4 (panel C) and αPgp-L8 (panel E) stained predominantly the plasma membrane of CH^RB30 cells. No signal was detected on plasma membranes in control experiments with either normal mouse IgG (panel B), or preimmune sera of αPgp-L4 (panel D) or αPgp-L8 (panel F). Under the same condition, no staining was observed with the parental Aux B1 cells (data not shown), consistent with the results on Western blot shown in Fig. 1.

6.2.2. Expression of multiple topologies of Pgp in multidrug resistant CH^RB30 cells:

Two methods were used to determine the membrane orientation of Pgp in drug-resistant CHO cells: proteolysis/membrane protection assay and immunocytochemistry as described in 6.1.2. Figs. 3A and 3B show the two possible topological models of Pgp and the predicted protease-resistant fragments (see boxes) which react with mAb C219, pAb αPgp-L4 and αPgp-L8. In the outside-out vesicles (Fig. 3A), both ATP-binding domains will be protected from trypsin digestion for both model I and model II Pgp molecules and protease-resistant fragments of ~44 and 43 kDa will be generated. These two fragments are expected to react with mAb C219. However, the fragments containing loops 4 (12 kDa) and 8 (23 kDa), which can be detected by pAbs αPgp-L4 and αPgp-L8, respectively, will be generated only from the model I Pgp molecules by trypsin cleavage at the extracellular loops (as indicated by solid triangles in Fig. 3A). In the model II molecules, these two loops will be completely digested by trypsin due to the existence of a large number of trypsin-cleavage sites and, therefore, no fragments from the model II structure will be detected by pAb αPgp-L4 and αPgp-L8. In the inside-out vesicles

(Fig. 3B), on the other hand, both ATP-binding domains in both model I and II Pgp molecules are on the outside of vesicles and hence will be digested by trypsin. Loops 4 and 8 in the model I molecules will also be digested by trypsin. However, loops 4 and 8 in the model II molecules will be protected from trypsin digestion and fragments containing TM segments and these two loops (16 or 20 kDa for loop 4 and 19 kDa for loop 8) will be detected by pAb α Pgp-L4 and α Pgp-L8 (Fig. 3B). The fragments containing putative loops 4 and 8 generated from inside-out and outside-out vesicles will have different sizes.

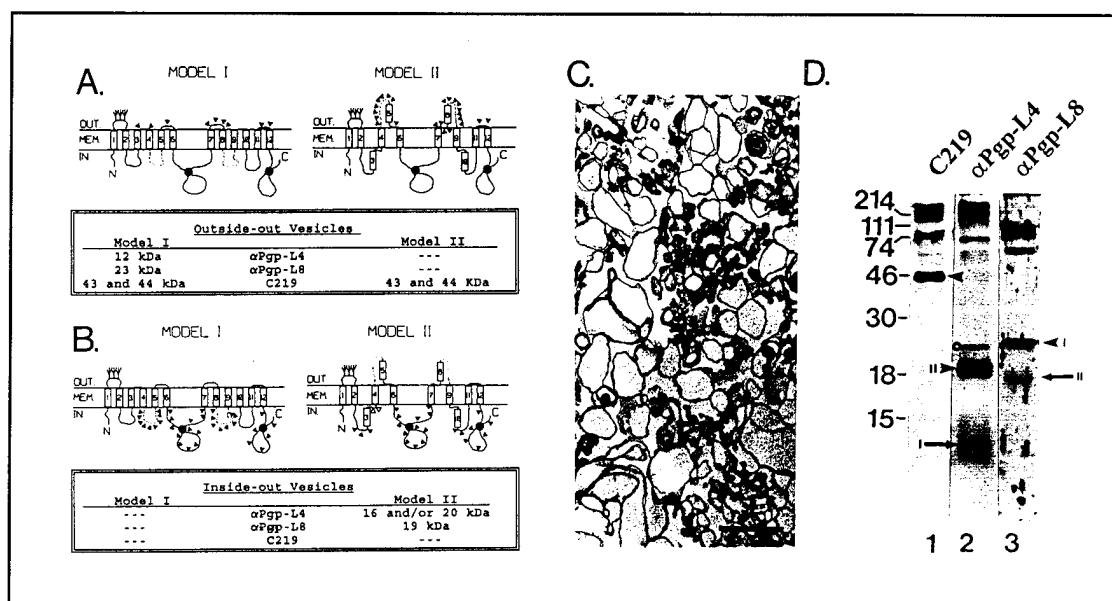


Figure 3. Analysis of P-glycoprotein topology using proteolysis/membrane protection assay. *A* and *B*, predicted proteolysis of Pgp in outside-out and inside-out vesicles. Both model I and II Pgp in outside-out (*A*) and inside-out (*B*) vesicles and predicted fragments from trypsin digestion are presented. The putative TM segments are shown as numbered open bars. Relevant potential trypsin cleavage sites (Arg and Lys) on the outside of membrane vesicles are shown by triangles. Putative loops 4 and 8 are indicated by dashed lines. The predicted fragments protected from proteolysis, which can be detected by C219, α Pgp-L4, and α Pgp-L8, are given in the box below each model. *C*, integrity of the membrane vesicles isolated from CH^RB30 cells. Membrane vesicles were isolated from CH^RB30 cells and prepared for electron microscopy. A typical view of membrane-vesicle preparation was shown. The bar denotes 1 μ m. *D*, Western blot of Pgp fragments generated from trypsin treatment. Complete trypsin digestion of crude membranes from CH^RB30 cells was performed. mAb C219 detects fragments of 44 kDa (lane 1). The antibody α Pgp-L4 recognized a 19-kDa fragment indicated by an arrowhead and a 11-12 kDa band indicated by an arrow (lane 2). The antibody α Pgp-L8 reacted with two fragments of 24 kDa (indicated by an arrowhead) and 18 kDa (indicated by an arrow) in lane 3.

Fig. 3C shows a representative view under electron microscopy of sealed crude membrane vesicles prepared from CH^RB30 cells. The membrane sidedness of isolated membrane vesicles was determined by testing the activity of ecto-acetylcholinesterase in the

absence and presence of 0.1% Triton X-100 to permeabilize the membranes. The difference in activity of acetylcholinesterase between these two treatments represents sealed inside-out vesicles. About 50% of the crude-membrane-vesicles prepared in this study have an inside-out orientation.

Fig. 3D shows the Western blot of fragments from the complete proteolysis of membrane vesicles with mixed orientations detected by mAb C219 and pAb α Pgp-L4 and α Pgp-L8. Various fragments protected from trypsin digestion were generated and detected by the antibodies. The monoclonal antibody C219 detected fragments of ~44 kDa (lane 1, Fig. 3D) which presumably represent the ATP-binding domains protected from digestion (see Fig. 3A). α Pgp-L4 detected a major fragment of 19 kDa (indicated by an arrowhead in lane 2, Fig. 3D) which presumably represents the fragments consisting of TM4-L4-TM5-L5-TM6 of the model II structure in the inside-out vesicles (see Fig. 3B). A band of ~11 kDa was also detected by α Pgp-L4 (indicated by an arrow in lane 2, Fig. 3D). This band may represent the TM4-L4-TM5 fragment of the model I structure in the outside-out vesicles. The arginine and lysine residues between the putative TM3 and TM4 in the model II structure may be covered by the putative TM3 located outside the membrane bilayer and, therefore, are not accessible to trypsin (see the open triangle indication between TM3 and TM4 in the model II structure in Fig. 3B). On the other hand, these two residues in the model I structure in the outside-out vesicles are exposed and freely accessible to trypsin (solid triangles in the model I structure in Fig. 3A). This may explain why only 12- and 20-kDa peptide fragments and no 16-kDa fragments (representing TM4-L4-TM5-L5-TM6 of the model II structure in the inside-out vesicles, Fig. 3B) were detected by α Pgp-L4. A major fragment detected by α Pgp-L8 is about 24 kDa (indicated by an arrowhead in lane 3, Fig. 3D). It probably represents the fragment of TM8-L8-TM9-L9-TM10-L10-TM11 of the model I structure in the outside-out vesicles (Fig. 3A). A minor band of ~18 kDa was also detected by α Pgp-L8 (indicated by an arrow in lane 3, Fig. 3D). It presumably represents the fragment of TM7-L7-TM8-L8-TM9-L9-TM10 of model II structure in the inside-out vesicles (Fig. 3B). Similar membrane-protected fragments were also generated by proteinase K digestion and detected by α Pgp-L4 and α Pgp-L8 (data not shown). These results suggest that both model I and II structures of Chinese hamster *pgp1* Pgp are likely present in isolated membranes from CH^RB30 cells. Indirect whole-cell staining using immunocytochemistry with the pAb α Pgp-L4, α Pgp-L8, and mAb C219 also showed that both model I and II structures of Chinese hamster *pgp1* Pgp are expressed (data not shown).

6.2.3. A peptide fragment including TM11 and TM12 of human MDR1 Pgp was expressed in the plasma membrane of *E. coli* BL21/DE3: A cDNA fragment encoding the putative TM11-TM12 and their flanking regions of human *MDR1* Pgp has been cloned into the expression vector pET-16B. The newly generated plasmid is name pET-MDR1. Pgp peptide fragment containing TM11, TM12, and the epitope of monoclonal antibody C494 specific to Pgp has been expressed in the *E. coli* BL21/DE3. Fig. 4 shows a Western blot of the plasma membrane fraction from the bacteria transformed with the vector pET-16B (lane 2) or transformed with pET-MDR1 (lane 1). It is clear that a protein of ~27 kDa was produced in the bacteria with the recombinant Pgp cDNA upon induction by IPTG (lane 2). Currently, the topology of this peptide fragment in membrane and its activity in binding anticancer drugs are being determined.

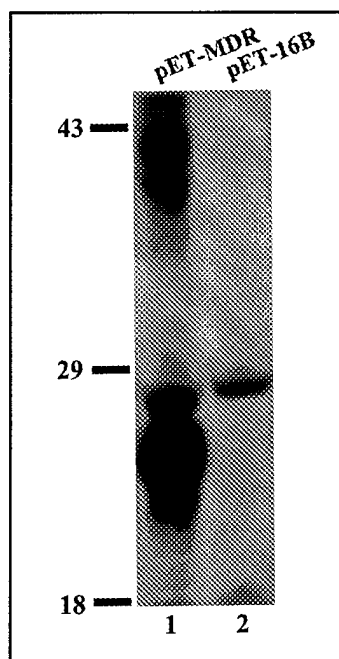


Figure 4. Expression of TM11 and TM12 in bacteria. A cDNA fragment (Nde I-Ban II) of human MDR1 encoding the TM11, TM12, and their surrounding sequences containing a C494 epitope was cloned into pET-16B to generate pET-MDR1. *E. coli* transformed with pET-16B and pET-MDR1 were induced by IPTG and plasma membranes were prepared for SDS-PAGE and Western blot probed with C494. The 27-kDa protein detected by C494 in lane 1 is presumably the Pgp fragment. A 28-kDa protein generated in both cells is also detected by C494. Its source is unknown. The higher molecular weight proteins produced only in pET-MDR1 cells (lane 1) are presumably the aggregated proteins of the Pgp fragment.

7. CONCLUSIONS:

In the past one year, we have determined the topologies of P-glycoprotein in multidrug resistant cells using site-specific antibodies. We found that P-glycoproteins in the plasma membrane of mammalian cells express at least two alternate topologies. This observation is consistent with our previous study using cell-free expression system. The more than one topology feature of Pgp may be responsible for its multifunctional nature. Two manuscripts on studies supported by the current award have been submitted for publication (see Appendix for the abstracts of these two manuscripts).

We have also been able to express the transmembrane domain of Pgp in bacteria. The putative TM11 and TM12 have been suggested to be drug-binding site (Zhang *et al.*, 1995b). By expressing the drug-binding domain alone in bacterial will provide an opportunity to prepare large quantity for further structure and function studies. For example, the 3-dimensional (3-D) fold of the drug-binding peptides can be analyzed using x-ray crystallography and NMR technology. Studies of 3-D structure will help define the group(s) of drugs that interact with the drug binding peptides. Alteration of the groups of these drugs may lead to a design of new agents that will overcome the MDR, but still have therapeutic effects. In the second year of this project, we will also test the hypothesis that the multiple topology of Pgp is responsible for its multifunctional nature. We will lock the Pgp in one topological orientation at a time using site-directed mutagenesis (Greenberger *et al.*, 1991; Bruggemann *et al.*, 1992; Zhang *et al.*, 1995a) and determine the function of the mutant Pgp.

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9. APPENDIX (Abstracts of manuscripts submitted for publication)

TITLE: Multiple topologies of P-glycoprotein in native plasma membranes of multidrug-resistant CH^RB30 cells: implications for the multifunctional nature of the molecule

AUTHORS: Mei Zhang and Jian-Ting Zhang

ABSTRACT: P-glycoprotein (Pgp) is an energy-dependent drug efflux pump expressed in the plasma membrane of tumor cells, and is responsible for multidrug resistance (MDR) in cancer cell lines. Pgp has also been suggested to have other functions, such as (1) a volume-regulated Cl⁻ channel, (2) a regulator of Cl⁻ channel, (3) an ATP-conducting channel, (4) a peptide transporter, and (5) an intracellular pH regulator. Moreover, coexpression of multiple topologies for Pgp have been observed in a cell-free expression system [Zhang, J.T., Duthie, M., and Ling, V. (1993) *J. Biol. Chem.* **268**, 15101-15110]. The current study was designed to determine the topology of Pgp naturally expressed in mammalian MDR cell plasma membranes. We generated site-specific antibodies against the loop linking putative TM4 and TM5 (loop 4) and the loop linking putative TM8 and TM9 (loop 8) of Pgp. These antibodies were then used as probes to analyze the membrane orientation of Pgp in multidrug-resistant CH^RB30 cells. Using the Western blot technique combined with the proteolysis/membrane protection assay of isolated membranes, and immunocytochemical staining of whole cells, we were able to show that Pgp expressed in the multidrug-resistant CH^RB30 cells has more than one topological structure. These findings are the first demonstration of multiple topologies of a native plasma membrane protein. These *in vivo* studies, together with previous observation in a cell-free system, indicate that Pgp is a unique molecule whose multiple topologies may be responsible for its multiple functions. Furthermore, the multiple topologies may provide an underlying mechanism for the drug-transport function in MDR.

TITLE: Co-translational Effects of Temperature on Membrane Insertion and Orientation of P-glycoprotein Sequences

AUTHORS: Jian-Ting Zhang and Crispina H. Chong

ABSTRACT: P-glycoprotein (Pgp) is a membrane transport protein that causes multidrug resistance (MDR) by actively extruding a wide variety of cytotoxic agents out of cells. It may also function as a peptide transporter, a volume-regulated chloride channel, and an ATP channel. Previously, it has been shown that hamster *pgp1* Pgp is expressed in more than one topological form and that the generation of these structures is modulated by charged amino acids flanking the predicted transmembrane (TM) segments 3 and 4 and by soluble cytoplasmic factors. Different topological structures of Pgp may be related to its different functions. In this study, we examined the effects of translation temperature on the membrane insertion process and the topologies of Pgp. Using the rabbit reticulocyte lysate expression system, we showed that translation at different temperatures generated different membrane orientations of the putative TM3 and TM4 of hamster *pgp1* Pgp in a co-translational manner. This observation suggests that the membrane insertion process of TM3 and TM4 of Pgp molecules may involve a protein conducting channel which acts in a temperature sensitive manner. Our observation may provide a way to understand the structure-function relationship of Pgp and may help overcome Pgp-related multidrug resistance of cancer cells.